REMARKS

The Rejection Under 35 USC § 112, first paragraph

The Office Action alleges that claims 1,9,11, 12, 13, 15, 16, 37, 41-43, 46, 80 and 81 are not enabled.

The allegations however only appear to be directed to the method claims, while many of these claims, e.g., claims 37, 41-43, and 46, are compound claims. Clarification as to why the compound claims were rejected is respectfully requested.

The Office Action cites in view of Lemoine et al., in table 10.2 lists ras oncogene mutations in small-cell lung carcinoma (SCLC) as being of low-frequency. However, this does not mean to one of ordinary skill in the art that small-cell lung carcinoma cannot be treated by the ras pathway. Nothing in the reference states that such is the case.

Additionally, applicants attach a publication titled "Activated Raf-1 Causes Growth Arrests in Human Cell Lung Cancer Cells" which states on page 158, second column, that "data demonstrates that raf can function as a growth suppressor gene in SCLC."

The issue still remains that not all cancers respond equally to affects on the ras/raf pathway. However, that is no basis for an enablement rejection. The Office Action admits that raf/ras pathway is correlated to many cancers, some highly, some intermediately, and so on. However, the law is clear that in cases, even where a broad claim includes inoperative embodiments, such is not problematic because one of ordinary skill in the art would know how to avoid the same. See *In re Dinh-Nguyen*, 181 USPQ 46 (CCPA 1974), and *In re Sarett*, 140 USPQ 474 (CCPA 1964).

This issue has also long been decided in *Sarett*, which states that

> It is certainly not incumbent on an applicant who has made a broad ... invention and supported it by an adequately broad disclosure to demonstrate the operativeness of every substance falling within the scope of the broad claims to which he is entitled. ... The function of claims is to *point out* the invention and *define* the scope of the monopoly, not to exclude substances which are possibly of no use in practicing the invention. (Emphasis added.)

Dinh-Nguyen, states that

> It is *not a function of the claims* to specifically exclude either possible inoperative substances or ineffective reactant proportions. (Emphasis added.)

In the present case, studies on the raf/ras pathway and related cancers thereto are numerous; and thus, one of ordinary skill in the art would know how to proceed in cases

where inoperative embodiments may occur. Applicants invented a broad invention, which under the applicable law, does not have to be limited to specific embodiments since the invention is not so limited.

Applicants submit that the claims are enabled, and that one of ordinary skill in the art in view of what is known in the art, can, without under experimentation, practice the claimed invention.

For all the foregoing reasons, reconsideration is respectfully requested.

Additionally, Lemoine et al., as admitted in the Office Action, teaches that pancreatic cancer, acute myeloid leukemia, and colorectal cancer are highly associated with the ras/raf kinase pathway, while bladder cancer is associated with the ras/raf kinase pathway to an intermediate extent. Consideration of some of the new claims in view of this admission is respectfully requested.

The Rejection Under 35 USC § 112, second paragraph

The Office Action alleges that "up to per-halosubstitution" is indefinite in the claims and that it us unclear what this range is encompassing and what the lower limit of this range is. Applicants respectfully disagree.

The language appears in claim 1, as "optionally substituted by one or more substituents independently selected from the group consisting of halogen, up to per-halosubstitution." Since this is an optional substitution, it is clear that zero substitution is the lower limit. It is also clear that " up to per-..." means up to 4 subsitutions. Thus, one of ordinary skill in the art would understand this language to mean 0, 1, 2, 3 or 4 halogen subsituents. Changing the language to expressly state " 0, 1, 2, 3 or 4 halogen subsituents" merely would recite an equally clear alternate way to state the same, but would not further clarify the already clear original language. Thus, applicants decline to amend at this point.

In claims 37, 43 and 81 the substation is not optional; thus, one of ordinary skill in the art would understand the language to mean " 1, 2, 3 or 4 halogen subsituents," especially in light of the groups modified by the term are also separately recited in their non-substituted form.

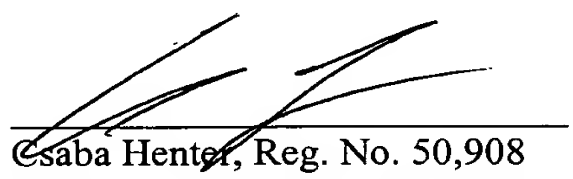
Reconsideration of this rejection is respectfully requested.

The Specification

The specification is amended to include the proviso from original claim 1.

The Commissioner is hereby authorized to charge any fees associated with this response or credit any overpayment to Deposit Account No. 13-3402.

Respectfully submitted,



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Activated Raf-1 Causes Growth Arrest in Human Small Cell Lung Cancer Cells

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Abstract

Small cell lung cancer (SCLC) accounts for 25% of all lung cancers, and is almost uniformly fatal. Unlike other lung cancers, ras mutations have not been reported in SCLC, suggesting that activation of ras-associated signal transduction pathways such as the raf-MEK mitogen-activated protein kinases (MAPK) are associated with biological consequences that are unique from other cancers. The biological effects of raf activation in small cell lung cancer cells was determined by transfecting NCI-H209 or NCI-H510 SCLC cells with a gene encoding a fusion protein consisting of an oncogenic form of human Raf-1 and the hormone binding domain of the estrogen receptor (ΔRaf-1:ER), which can be activated with estradiol. ΔRaf-1:ER activation resulted in phosphorylation of MAPK. Activation of this pathway caused a dramatic loss of soft agar cloning ability, suppression of growth capacity, associated with cell accumulation in G1 and G2, and S phase depletion. Raf activation in these SCLC cells was accompanied by a marked induction of the cyclin-dependent kinase (cdk) inhibitor $p27^{kip1}$, and a decrease in cdk2 protein kinase activities. Each of these events can be inhibited by pretreatment with the MEK inhibitor PD098059. These data demonstrate that MAPK activation by ΔRaf-1:ER can activate growth inhibitory pathways leading to cell cycle arrest. These data suggest that raf/MEK/MAPK pathway activation, rather than inhibition, may be a therapeutic target in SCLC and other neuroendocrine tumors. (*J. Clin. Invest.* 1998. 101:153–159.) Key words: SCLC • activated raf • MAP kinase • cell cycle • $p27^{kip1}$ • MEK inhibitor PD098059

Introduction

c-raf-1 is a cytosolic serine/threonine protein kinase that is central to several intracellular signal transduction pathways. Activated ras can translocate raf to the cell membrane where it is activated by a process possibly involving tyrosine phosphorylation (1–4). Upon activation, c-raf-1 phosphorylates MEK, activating downstream mitogen-activated protein kinases (MAPK[1]/ERKs). This phosphorylation cascade leads to the activation of transcription factors involved in cell growth and differentiation (5). Raf and mutated forms of ras act as dominant oncogenes, and can collaborate with other oncogenes to transform primary cells. In human lung cancers, activating ras mutations are common in adenocarcinomas and squamous cell cancers, but are not observed in small cell lung cancer (SCLC) (6). We previously showed that insertion of a mutated *ras* gene could differentiate some SCLC cell lines (7). These observations taken together suggested that activation of MAPK by ras mutation or raf activation might have growth suppressive activity in SCLC. We show here that activation of an estradiol-regulated form of human c-raf-1 (8) in SCLC cells can suppress growth by causing SCLC cells to become blocked in G1 and G2 of the cell cycle, unlike in other systems where raf activation results in transformation. This cell cycle arrest is associated with induction of the cyclin-dependent kinase (cdk) inhibitor $p27^{kip1}$, occurs irrespective of whether p53 is mutated. Our findings indicate that activation of the raf/MEK/MAPK pathway can cause SCLC cells to arrest in G1 and G2, accompanied by $p27^{kip1}$ induction.

Methods

Cell culture and cell lines. NCI-H209 and NCI-H510 human SCLC cell lines (9) were cultured in RPMI-1640 medium without Phenol red, 9% FBS (Sigma Chemical Co., St. Louis MO), 100 U/ml penicillin, 100 μg/ml streptomycin sulfate (Gibco, Grand Island, NY) in a humidified atmosphere of 5% CO_2 at 37°C. Cells were infected with equal volumes of retroviral supernatant from PA317-producer cells transfected with a retroviral vector pLNCX containing the activatable ΔRaf-1:ER fusion construct. Infection of SCLC cells was augmented by 2 μg/ml polybrene (Sigma Chemical Co.) in the medium. After 48 h, the medium was replaced by selection medium containing 0.5 mg/ml of G418. Pooled cultures of G418-resistant cells were grown, total RNA was extracted and analyzed for the presence of ΔRaf-1:ER by Northern blot analysis. SCLC cells expressing ΔRaf-1:ER were treated with 1 μM β-estradiol to activate the ΔRaf-1:ER fusion molecule. Untransfected Parent cells, parental cells exposed to 1 μM β-estradiol, and cells transduced for ΔRaf-1:ER but unexposed to β-estradiol were used as controls. No effects of ΔRaf-1:ER transduction in the absence of estradiol were observed.

Soft-agar cloning assay. Soft agar cloning assays were performed in 35-mm dishes over a bottom layer of 0.8% low-melting agarose in growth medium and 1.5×10^4 cells were plated in growth media containing 0.4% (wt/vol) agarose in the presence or absence of 1 μM estradiol. After 3 wk of incubation in a humidified atmosphere con-

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1. *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; cdk, cyclin-dependent kinase; MAPK, mitogen-activated protein kinase; SCLC, small cell lung cancer.

taining 5% CO_2 at 37°C, colonies with > 30 cells were scored, and percent cloning efficiency was calculated.

Cell cycle analysis. Cells were washed with ice cold 0.2% BSA in PBS, suspended in sucrose/citrate buffer (10). Nuclei were prepared, stained with propidium iodide and analyzed by an EPICS 752 flow cytometer (Coulter Electronics, Hialeah, FL) with a gate that selects single nuclei within a normal size range. The cell cycle parameters from 10,000 gated nuclei were determined by multicycle software (Phoenix Flow Systems, San Diego, CA). In some experiments, cells were pulse labeled with 1 μM bromodeoxyuridine (BrdU; Sigma Chemical Co.) for 45 min at 37°C and were fixed in 70% ethanol/PBS. Extracted nuclei were stained with FITC-labeled anti-BrdU antibodies (Becton Dickinson, San Jose, CA) and propidium iodide. Flow cytometry analysis was performed as described above.

Northern blotting. Total RNA was extracted with an acid phenol-guanidinium isothiocyanate method (11). Total RNA (20 μg/lane) was separated on 1.2% agarose/2.2 M formaldehyde-denaturing gels and transferred to Zeta-Probe (Bio-Rad, Melville, NY). Probes used in Northern analysis were ΔRaf-1:ER, ClaI-XhoI fragment of pLNCX ΔRaf-1:ER (8); and human β-actin, BamH1 fragment (kindly provided by Don Cleveland, Johns Hopkins University, Baltimore, MD). These probes were labeled with [α-^{32}P] dCTP (Dupont, New England Nuclear, Boston, MA) by random primer labeling (Boehringer Mannheim, Indianapolis, IN). Hybridizations were done using radiolabeled probe (1×10^6 cpm/ml) at 42°C for 16–18 h, then rinsed twice at room temperature and washed once for 30 min at 65°C with 1 × SSC and 1% SDS. Membranes were then exposed to x-ray film (Kodak X-0 MAT; Eastman Kodak Co., Rochester, NY) at −80°C with intensifying screens.

Western blotting. Cells were lysed in PBS, 1% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 2.5 mM PMSF. After protein concentrations were determined, 100 μg of proteins were resolved on SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were probed with anti–Raf-1 (C-12), anti-$p27^{kip1}$ (C-19), anticyclin E (HE12), anti-cdk2 (M2), anti-$p34^{cdc2}$ (C-17) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); anti-MAPK (phosphospecific MAPK from New England Biolabs, Beverly, MA). Immunoreactive protein complexes were detected by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL).

Kinase assays. Whole cell lysates were prepared in 50 mM Tris, pH 7.5, 137 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 50 mM β-glycerophosphate, 2 mM EDTA, 10 mM EGTA, 1 mM DTT, 1 mM Na_3Vo_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM phenylmethyl sulfonyl fluoride. 100 μg of whole cell lysates were incubated for 2 h with 1 μg/ml anti-cdk2 or anti-MAPK. The immune complexes were immunoprecipitated with protein A Sepharose beads (Pharmacia Fine Chemicals, Piscataway, NJ) for 1 h, and the immune complexes bound to the beads were washed with the same lysis buffer. Kinase assays were performed using histone H1 or myelin basic protein (MBP) (Sigma Chemical Co.), for cdk2 or MAPK, respectively. For kinase assays, the immunoprecipitates and substrates were incubated in a volume of 40 μl containing 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.125 μCi/μl γ-^{32}P ATP at 30°C for 20 min. Substrates for these kinase assays were either 20 μg histone H1 for cdk2 assays, or 20 μg myelin basic protein for MAP kinase assays. The reactions were stopped by the addition of 40 μl of 2× Laemmli buffer, boiled for 3 min and resolved on 12.5% SDS-PAGE gels. Phosphorylated proteins were visualized by autoradiography and quantitated on a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Results

SCLC cells from cell lines NCI-H209 and NCI-H510 were transduced with a vector expressing ΔRaf-1:ER. This vector encodes a conditionally activatable human c-Raf-1 which is transforming in many cell systems (8), and can differentiate some cells

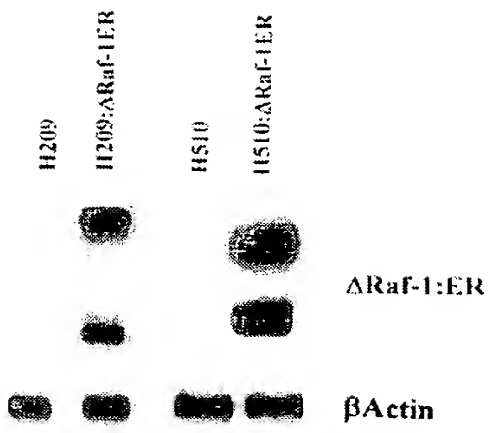


Figure 1. Northern analysis of ΔRaf–1:ER expression in SCLC cells. Expression of ΔRaf–1:ER after the stable transfection of NCI-H209 and NCI-H510 cells with a retroviral vector PLNCX containing the activatable ΔRaf-1:ER fusion construct. Total RNA was extracted and 20 μg of RNA was electrophoresed through a 1.2% formaldehyde gel and transferred to a nylon membrane. Northern analyses was performed as described in Methods and expression of β-actin served as loading control.

(12, 13). ΔRaf-1:ER can be activated by the addition of 1 μM estradiol or 4-hydroxy-tamoxifen to cells that stably express this construct. Pooled proliferating G418-resistant cultures of each transduced SCLC cells were analyzed for the expression of ΔRaf-1:ER construct (Fig. 1). SCLC cells transfected with ΔRaf-1:ER were exposed to estradiol and their ΔRaf-1:ER–transduced counterparts without estradiol, as well as parental cells with and without estradiol, were used as controls. Activation of ΔRaf-1:ER resulted in the phosphorylation of endogenous Raf-1 and downstream MAPK in NCI-H209:ΔRaf-1:ER and NCI-H510:ΔRaf-1:ER cells (Fig. 2). To measure the duration of MAPK activation, we activated ΔRaf-1:ER for different periods of time and assessed the phosphorylation and enzymatic activity of MAPK. Phosphorylation of MAPK was similar in time periods from 24 h to 7 d after estradiol treatment; at the 4-h time point, phosphorylation of MAPK was very low (Fig. 3 *A*). Enzymatic activity of MAP kinase was also the same during this time period (Fig. 3 *B*). Estradiol activation of ΔRaf-1:ER resulted in markedly changed cellular morphology

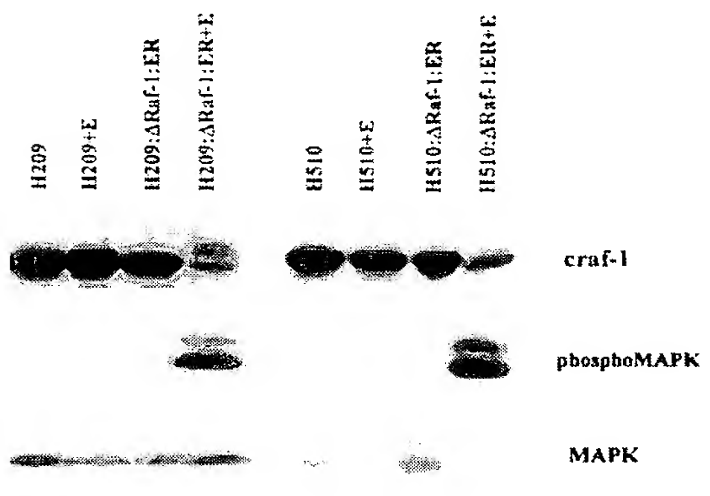


Figure 2. Activation of ΔRaf–1:ER caused the phosphorylation of endogenous c-Raf-1 and MAP kinase in SCLC cells. Lysates from parental SCLC cells, SCLC cells treated with estradiol (+*E*) for 48 h, NCI–H209–transduced SCLC cells and estradiol-activated ΔRaf-1:ER SCLC cells were immunoblotted with antibodies to Raf-1, phosphorylated MAPK and control MAPK.

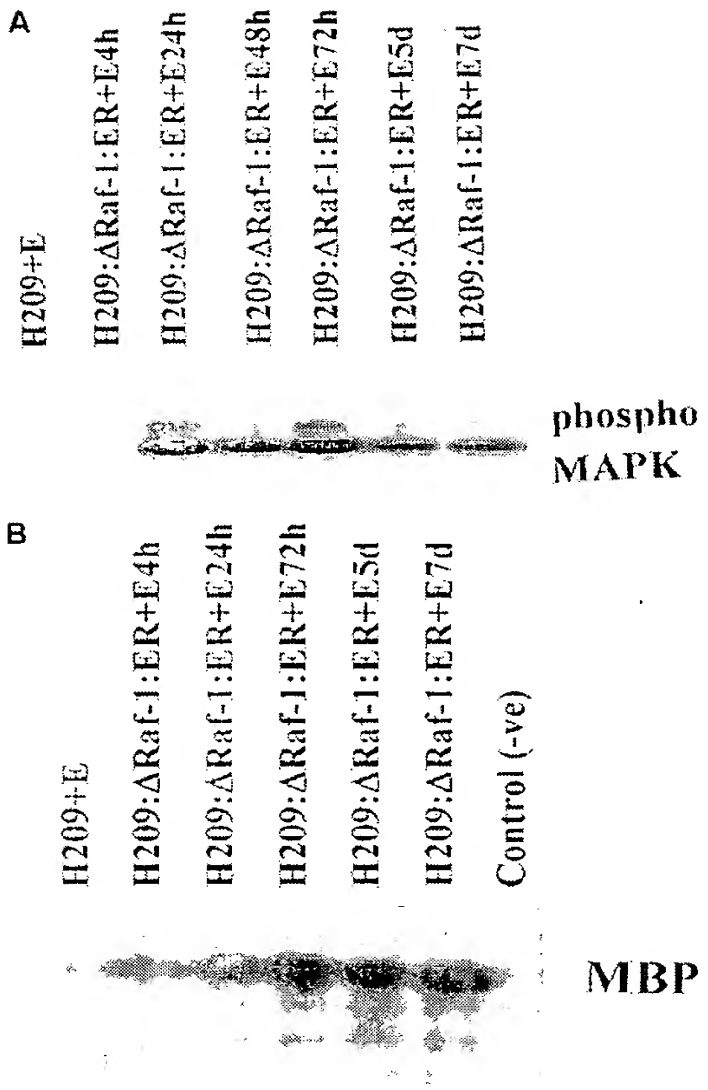
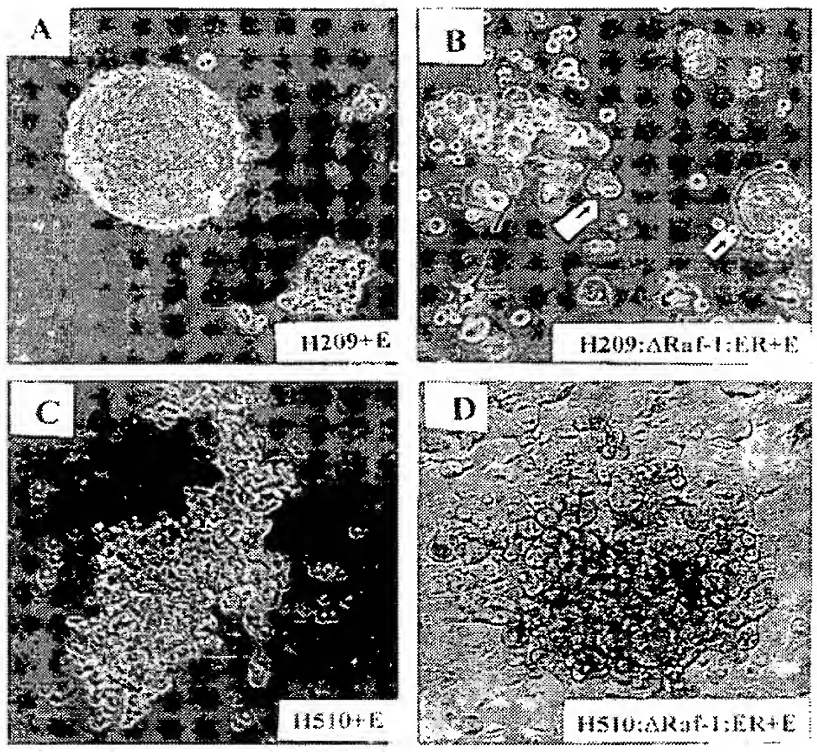


Figure 3. Phosphorylation and enzymatic activity of MAP kinase in ΔRaf-1:ER–activated SCLC cells. (*A*) Lysates from parental NCI-H209 cells and NCI-H209:ΔRaf-1:ER cells with activated ΔRaf-1:ER for different time periods were immunoblotted with phosphospecific MAPK antibody. (*B*) Whole cell lysates were immunoprecipitated with anti-MAPK and kinase activity was measured using myelin basic protein as a substrate as described in Methods. Phosphorylation and enzymatic activity of MAP kinase remained the same from 24 h to 7 d after ΔRaf-1:ER activation.

in NCI-H209:ΔRaf-1:ER cells and NCI-H510:ΔRaf-1:ER cells. Upon estradiol activation of ΔRaf-1:ER, these cells, which normally grow in suspension as floating suspended cell clusters (9), adhered to the plastic substratum of the tissue culture flasks, formed multinucleated giant cells, proliferated poorly, and virtually lost the ability to grow in soft agar (Fig. 4). This was not an effect of the endogenous estrogen receptor, since ΔRaf-1:ER activation by 4-hydroxy-tamoxifen (4-HT) in NCI-H209:ΔRaf-1:ER and NCI-H510:ΔRaf-1:ER cells had similar effects on growth, morphology, and cloning efficiency (data not shown). No effect of estradiol was observed on parental cells. We observed similar effects of ΔRaf-1:ER activation in NCI-



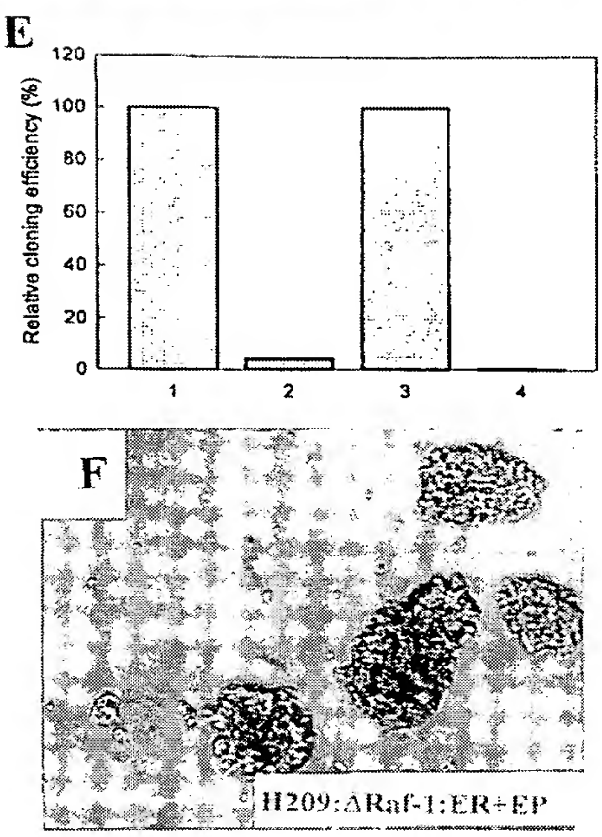


Figure 4. Morphological effects of activated ΔRaf-1:ER in NCI-H209 and NCI-H510 SCLC cells. Cells were grown in the presence of 1 μM estradiol (+*E*) for 6 d. Photographs of parental cells and transduced cells were taken using phase-contrast light microscope at a magnification of 100. No morphological changes were observed in parental cells grown in the absence or presence of estradiol. Parental cells exposed to estradiol (*A* and *C*) were used as controls for ΔRaf-1:ER maintain morphological characteristics as floating clusters of cells as do ΔRaf-1:ER–transduced cells without added estradiol. Activation of ΔRaf-1:ER caused the floating, suspended clusters of parental cells to become surface adherent and form multinucleated giant cells (*B* and *D*). (*E*) Activated ΔRaf-1:ER inhibited soft agar cloning. Soft agar cloning of NCI-H209(1), NCI-H209:ΔRaf-1:ER(2) NCI-H510(3), and NCI-H510:ΔRaf-1:ER(4) cells in the presence of 1 μM estradiol. (*F*) PD098059 blocked the morphological changes induced by activation of ΔRaf-1:ER. NCI-H209:ΔRaf-1:ER cells were treated with 10 μM PD098059 for 45 min before to the addition of 1 μM estradiol and grown for 72 h in the presence of PD098059 and estradiol (+*EP*) and photographed at a magnification of 100.

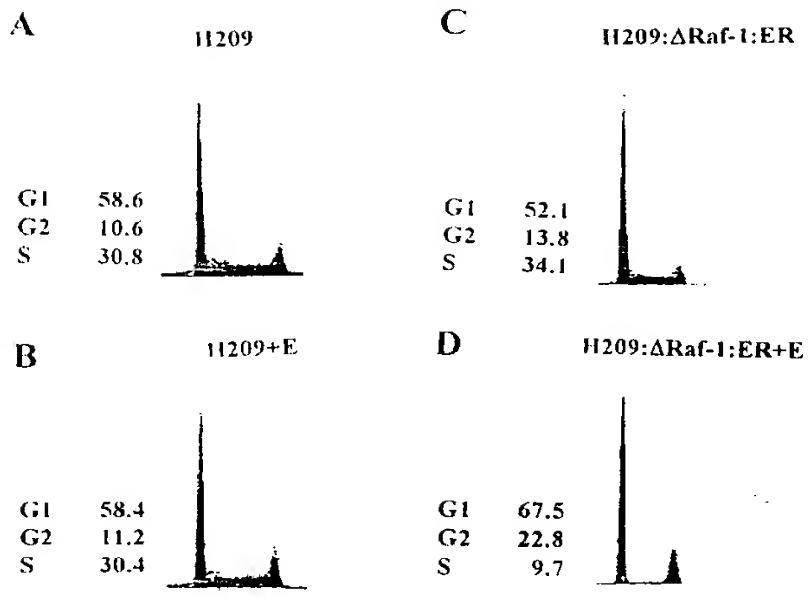


Figure 5. Effect of activated ΔRaf-1:ER on cell cycle distribution. SCLC NCI-H209 cells and their transduced ΔRaf-1:ER cells (*A* and *C*) were exposed to 1 μM estradiol (+*E*) for 6 d (*B* and *D*) and harvested for cell cycle distribution analysis using propidium iodide. Histograms show relative DNA content on x-axis and number of nuclei on the y-axis. Cell cycle distribution is similar to NCI-H209 and NCI-H510 cells after ΔRaf-1:ER activation.

H345, NCI-H69, and DMS53 SCLC cells (data not shown). The proportion of apoptotic cells (~ 5%) was not increased by ΔRaf-1:ER activation (data not shown), suggesting that the raf-induced growth suppression was due to inhibition of cell cycle progression.

Cell cycle analyses using propidium iodide and bromodeoxyuridine demonstrated cell cycle specific growth arrest upon ΔRaf-1:ER activation. ΔRaf-1:ER activation reduced the proportion of NCI-H209:ΔRaf-1:ER and NCI-H510:ΔRaf-1:ER cells in S phase and caused the cells to accumulate in both G1 and G2 (Fig. 5). No effects on cell cycle kinetics were observed in control NCI-H209 and NCI-H510 cells treated with estradiol, nor in ΔRaf-1:ER–transduced cells in the absence of estradiol. Cell cycle progression from G1 to S, measured by BrdU incorporation, was decreased by 79% in NCI-H209 cells, and by 77.3% in NCI-H510 cells upon ΔRaf-1:ER activation (Fig. 6). Karyotypic analysis failed to detect any metaphases in estradiol treated NCI-H209:ΔRaf-1:ER and NCI-H510:ΔRaf-1:ER cells (unpublished results).

Since cyclins and their cdks regulate the G1/S and G2/M transitions in the mammalian cell cycle (14), we examined whether cyclin–cdk complexes are altered in the cell cycle blocked SCLC cells after ΔRaf-1:ER activation. Members of the cyclin D family act in mid G1 by complexing with either cdk4 or cdk6 (15, 16), while cyclin E acts in late G1 by complexing with cdk2 (17, 18). As with most SCLC cells, NCI-H209 cells and NCI-H510 cells both lack a functional retinoblastoma susceptibility gene (Rb) (19, 20); p53 is mutated in NCI-H510 cells, and is wild-type in NCI-H209 cells (21). In pRb-defective cells, transition from G1/S is regulated by cyclinE–cdk2 complexes, but not by cyclin D–cdk4 or cyclin D–cdk6 (15, 16). Previous work has shown that cyclin D family members are poorly expressed in SCLC cells (22). Consistent with this observation, we were unable to detect cyclins D1, D2, or D3 in NCI-H209 and NCI-H510 cells with or without activated ΔRaf-1:ER. Cyclin E, however, was actually induced after ΔRaf-1:ER activation over the same time interval that NCI-H510 and NCI-H209 cells undergo cell cycle arrest (Fig. 7 *A*). Analysis of cdk2 expression by Western blotting demonstrated that neither activation of ΔRaf-1:ER nor treatment of the parental NCI-H209 and NCI-H510 cells with estradiol had any influence on cdk2 protein levels. Although cyclin E and cdk2 protein levels were not diminished in SCLC cells after ΔRaf-1:ER activation, the enzymatic activity of cdk2 was markedly reduced. Activation of ΔRaf-1:ER in SCLC cells reduced cdk2 kinase activity, as measured by histone H1 phosphorylation, by 91% in NCI-H209 cells and 78% in NCI-H510 cells (Fig. 7 *B*). Since it has been shown previously that cyclin E–cdk2 activity is necessary for S phase progression (23), these data suggest that ΔRaf-1:ER activation may block G1-S progression by reducing cdk2-associated kinase activity.

Decreased cyclin–cdk2 activity can result from expression of the cdk inhibitors $p21^{Cip1/Waf1}$ or $p27^{kip1}$, which bind cyclin–cdk complexes and inhibit their kinase activity. Overexpression of these cdk inhibitors has been shown to cause G1 arrest in several cell types (24). We were unable to detect $p21^{Cip1/Waf1}$ in NCI-H510:ΔRaf-1:ER cells or NCI-H209:ΔRaf-1:ER' cells, whether or not ΔRaf-1:ER was activated, nor by exposure of NCI-H510 and NCI-H209 cells to estradiol (data not shown). In contrast, $p27^{kip1}$ protein was induced by ΔRaf-1:ER activation in NCI-H510:ΔRaf-1:ER and NCI-H209:ΔRaf-1:ER

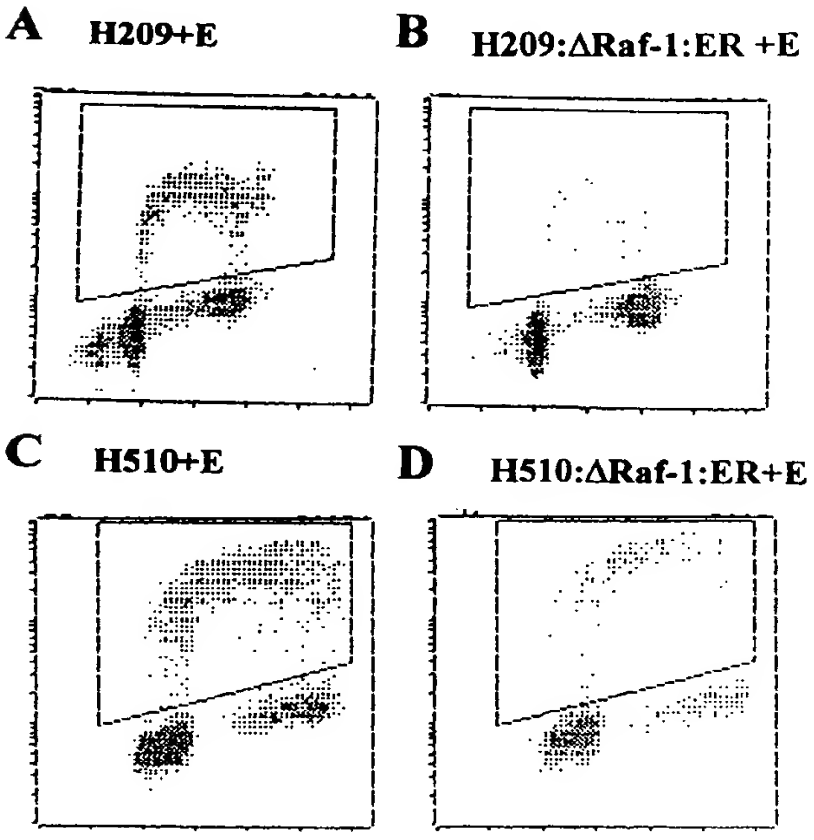


Figure 6. BrdU labeling of SCLC cells. SCLC control cells and their transduced ΔRaf-1:ER cells were grown in the presence of estradiol (+*E*) and were pulsed with BrdU. BrdU incorporation was measured using FITC-labeled anti-BrdU. Bitmaps were set using FITC-unlabeled cells. Histograms representing the BrdU labeling (y-axis) vs. the DNA staining (x-axis) and percentage of BrdU incorporated cells in S phase were measured using 10,000 cells.

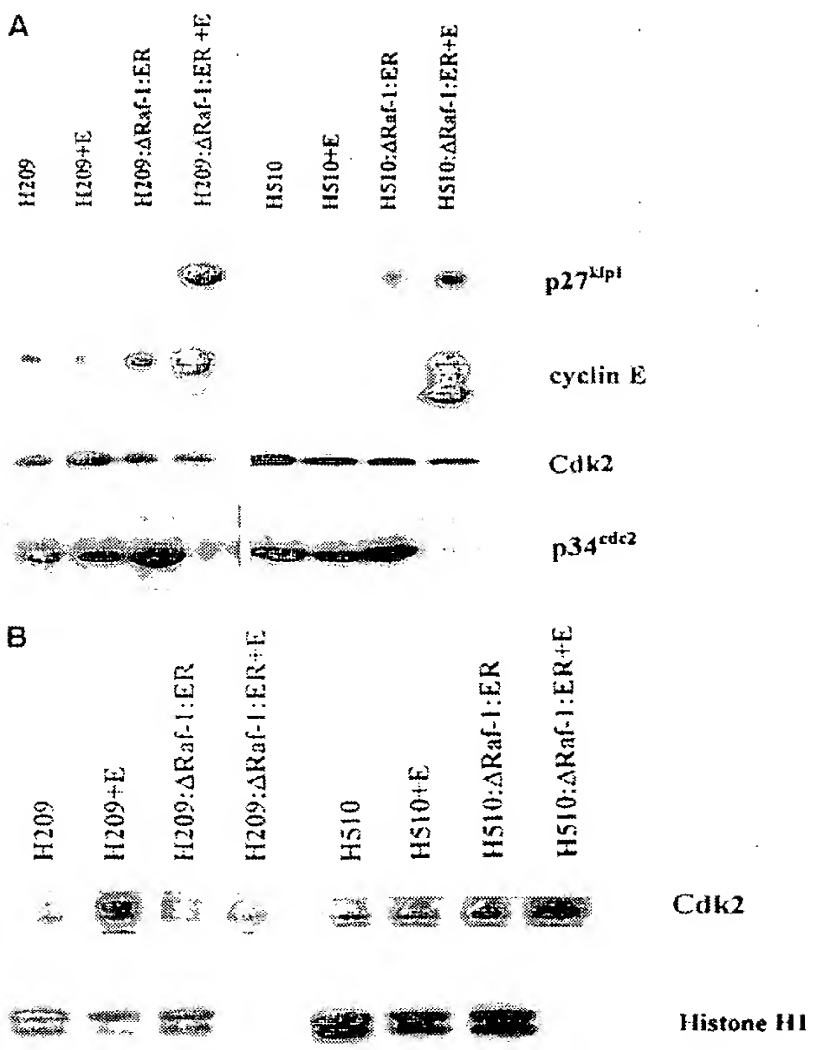


Figure 7. (*A*) Activation of ΔRaf-1:ER induced the expression of cyclin E, cdk inhibitor $p27^{kip1}$, reduced $p34^{cdc2}$ protein, and cdk2 kinase activity. Parental cells and their ΔRaf-1:ER–expressing cells were grown in the presence (+*E*) or absence of 1 μM estradiol for 6 d and harvested for proteins. Western blot analysis demonstrated that $p27^{kip1}$ is induced 20-fold in NCI-H209 cells and ninefold in NCI-H510 cells after ΔRaf-1:ER activation, compared with control cells. Expression of cyclin E (50–52 kD) was also increased in cells expressing activated ΔRaf-1:ER. In contrast, ΔRaf-1:ER activation markedly reduced $p34^{cdc2}$ protein in these SCLC cells. No effects on cdk2 protein expression were observed after ΔRaf-1:ER activation. (*B*) cdk2 activity immunoprecipitated from SCLC cells was measured using histone H1 as substrate. Activation of ΔRaf-1:ER led to decreased cdk2 kinase activity in NCI-H209:ΔRaf-1:ER cells by 91 and 78% in NCI-H510:ΔRaf-1:ER cells, compared with their untreated control cells. cdk2 protein levels from cdk2 immunoprecipitates were also shown.

cells, but was not evident in NCI-H510 and NCI-H209 control cells with or without estradiol (Fig. 7 *A*). Northern blot analyses showed that $p27^{kip1}$ mRNA levels were not altered after ΔRaf-1:ER activation (data not shown), consistent with previous data that $p27^{kip1}$ can be posttranscriptionally regulated (25).

Since both NCI-H209:ΔRaf-1:ER and NCI-H510:ΔRaf-1:ER SCLC cells also arrested in G2 after ΔRaf-1:ER activation, we examined whether mediators of the G2 transition were also modified in these cells. It has been shown that $p34^{cdc2}$ (cdc2) association with cyclin B to form a complex with kinase activity is essential for the G2/M transition (26), and that this molecule is present at fairly constant levels throughout the cell cycle. Activation of ΔRaf-1:ER in NCI-H209:ΔRaf-1:ER and NCI-H510:ΔRaf-1:ER cells strongly reduced $p34^{cdc2}$ protein expression (Fig. 7 *A*), suggesting that the G2 block in these cells may be due to interference with cyclin B–cdc2 activity. Since $p27^{kip1}$ also inhibits $p34^{cdc2}$ activity (27, 28), our data suggest that $p27^{kip1}$ may contribute to both the G1 and G2 blocks we have observed.

The ΔRaf-1:ER–dependent cell cycle block we observed resulted from activation of the MEK–MAPK signal transduction pathway. To determine this, we treated NCI-H510 and NCI-H209 cells transduced with ΔRaf-1:ER and controls with the MEK inhibitor, PD098059. PD098059 selectively blocks the activation of MEK, and thereby inhibits phosphorylation and activation of MAP kinases in vitro (29, 30). Exposure of NCI-H510:ΔRaf-1:ER and NCI-H209:ΔRaf-1:ER cells to 10 or 100 μM PD098059 for 45 min before ΔRaf-1:ER activation inhibited phosphorylation and activation of MAPK (Figs. 8 and 9). PD098059 allowed NCI-H209 and NCI-H510 cells with estradiol activated ΔRaf-1:ER to proliferate, and abolished cell accumulation in G1and G2 after ΔRaf-1:ER activation. Further, PD098059 reversed the morphological changes induced by ΔRaf-1:ER activation in SCLC cells (Fig. 4 *F*). Similarly, PD098059 prevented the induction of cyclin E and $p27^{kip1}$, and the reduced levels of $p34^{cdc2}$ that follow ΔRaf-1:ER activation in SCLC cells (Fig. 9). Also our finding is in accordance with another study that MEK inhibitor PD098059 can inhibit the ΔRaf-1:ER–induced $p21^{cip1}$ cdk inhibitor (31). There was no discernible effect of PD098059 on parental NCI-H209 and NCI-H510 cells (data not shown).

Our data indicate that ΔRaf-1:ER–mediated activation of MAPK inhibits the growth of SCLC cells, causing these cells to accumulate in the G1 and G2 phase of the cell cycle. This appears to be due in part to the induction of the cdk inhibi-

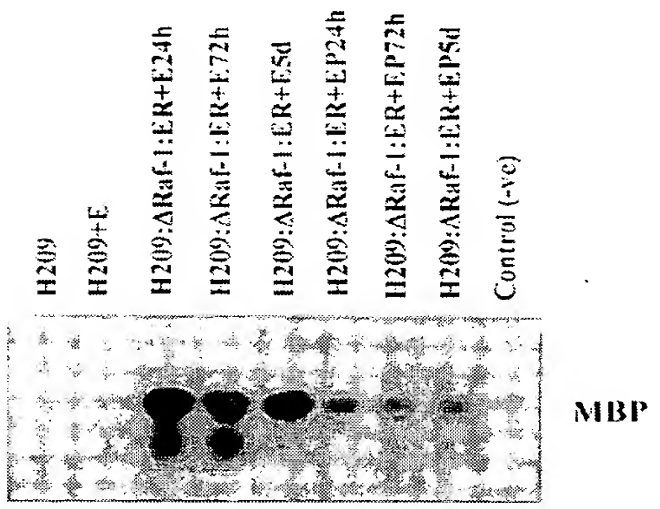


Figure 8. PD098059 inhibited the phosphorylation of MAPK. NCI-H209:ΔRaf-1:ER cells were treated with 10 μM PD098059 for 45 min before to the addition of 1 μM estradiol and grown for 24, 72 h, and 5 d in the presence of PD098059 and estradiol (+*EP*). PD098059-treated cell lysates and control cell lysates were immunoprecipitated with anti-MAPK and kinase assays were performed using MBP as substrate. ΔRaf-1:ER–activated NCI-H209:ΔRaf-1:ER cell lysate was used for negative control and kinase assay was performed without primary antibody. PD098059 inhibited the phosphorylation of MBP by 60% in ΔRaf-1:ER activated NCI-H209:ΔRaf-1:ER cells.

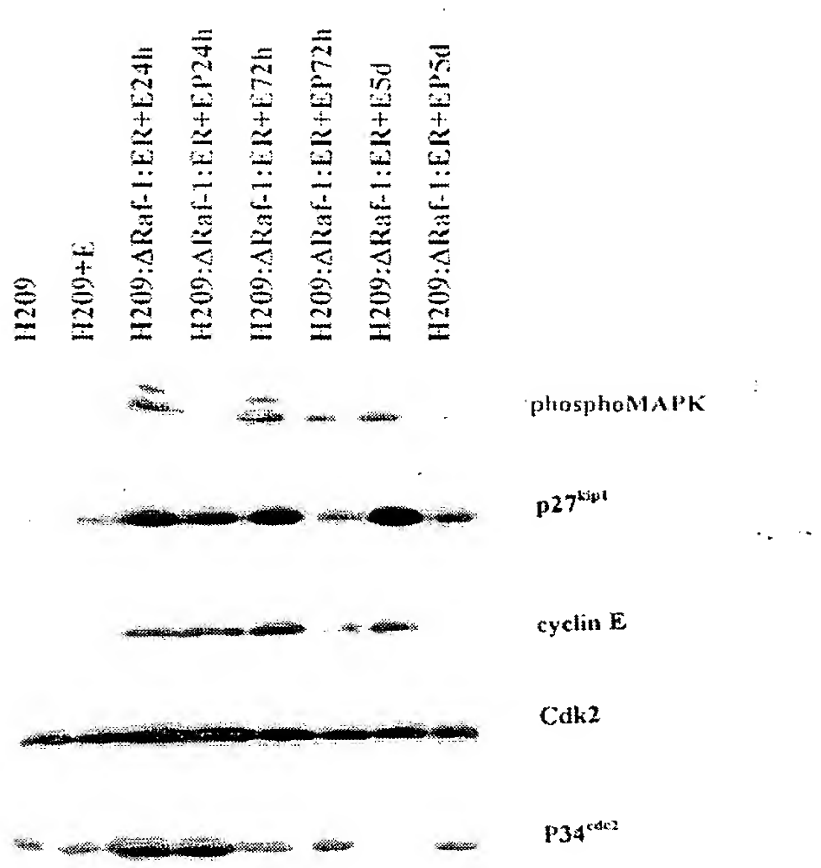


Figure 9. PD098059 inhibited the phosphorylation of MAPK, blocked the induction of $p27^{kip1}$, cyclin E, and restored $p34^{cdc2}$ levels in ΔRaf-1:ER–activated cells. NCI-H209:ΔRaf-1:ER cells were treated with 10 μM PD098059 for 45 min before the addition of 1 μM estradiol and grown for 5 d in the presence of PD098059 and estradiol (+*EP*). Western blots were made from the cell lysates of NCI-H209:ΔRaf-1:ER cells exposed to PD098059 and estradiol together (+*EP*) and estradiol alone (+*E*) for different time intervals and probed with phosphospecific MAPK antibody, anti-$p27^{kip1}$, anti–cyclin E, anti-cdk2, and anti-$p34^{cdc2}$, respectively.

tor $p27^{kip1}$ and its inactivation of cdk2. We examined whether $p27^{kip1}$ was responsible for the inactivation of cdk2 by use of a $p27^{kip1}$ inhibitor in cell extracts. $p27^{kip1}$ can be inactivated by adenovirus protein E1A, resulting in restoration of cdk2 activity (32). We tested, therefore, whether addition of E1A to cell extracts from NCI-H209:ΔRaf-1:ER and NCI-H510:ΔRaf-1:ER, to inactivate the induced $p27^{kip1}$, would restore cdk2 activity in these cell extracts. As shown in Fig. 10, E1A restored cdk2 activity to 48% of control levels in 209:ΔRaf-1:ER cells and to 26% of controls in 510:ΔRaf-1:ER cells. This indicates that $p27^{kip1}$ contributes significantly to the loss of cdk2 activity, and further suggests that $p27^{kip1}$ contributes significantly to the cell cycle block observed after ΔRaf-1:ER activation.

Discussion

The Raf/MEK/MAPK pathway has been shown to contribute to the transformation of primary cells and stimulation of cellular proliferation (1–3). In some cell types the Raf/MEK/MAPK pathway leads to cell differentiation (33, 12, 13). Our results show that ΔRaf-1:ER activation inhibits the growth of SCLC cells and is associated with the accumulation of cells in the G1 and G2 phases of the cell cycle. Our data suggest that this cell cycle block occurs via induction of the cdk inhibitor $p27^{kip1}$, leading to reduced cdk2 activity. Previous studies have shown that $p27^{kip1}$ inhibits a wide array of cyclin–cdk complexes, and that overexpression of $p27^{kip1}$ causes many cells to arrest in G1 (27, 28). Our data are similar to those observed when TGF-β induces cell cycle arrest in some systems (34, 35). However, addition of TGF-β to NCI-H510 and NCI-H209 cells only modestly increased $p27^{kip1}$ levels, and was unable to influence cdk2 activity or modify the cell cycle (data not shown). The addition of conditioned medium from the activated ΔRaf-1:ER cells to NCI-H209 and NCI-H510 parental cells also failed to influence the cell cycle (data not shown). These data indicate that the effects of ΔRaf-1:ER activation and resultant MAPK phosphorylation in our SCLC cells are not dependent on autocrine production of TGF-β.

These data demonstrate that raf can function as a growth suppressor gene in SCLC, a common neuroendocrine cancer, by activating signal transduction pathways coupled to regulatory molecules of the cell cycle. A role for the Ras/Raf/MAPK pathway in growth arrest or cellular differentiation has been suggested by earlier studies. Ectopic expression of v-Ras causes growth inhibition in Schwann cells and REF52 cells (36, 37). Human medullary thyroid carcinoma cells (12, 38), pheochromocytoma (PC12) cells (33), and hippocampal neuronal cells (13) are differentiated by ras and raf. In PC12 cells, ras-regulated hypophosphorylation of pRb mediates growth inhibition and neuronal differentiation (39). Also recent data have suggested that the ras pathway is capable of inducing premature cell senescence after the induction of cdk ihibitor $p16^{INK4a}$ (40). Our findings that activation of MAPK by ΔRaf-1:ER causes SCLC cells to arrest in the cell cycle extend these concepts, and also indicate that MAPK can activate cdk inhibitors such as $p27^{kip1}$ that can function to reduce cdk2 activity. Our findings suggest consideration of members of the raf/MEK/MAP

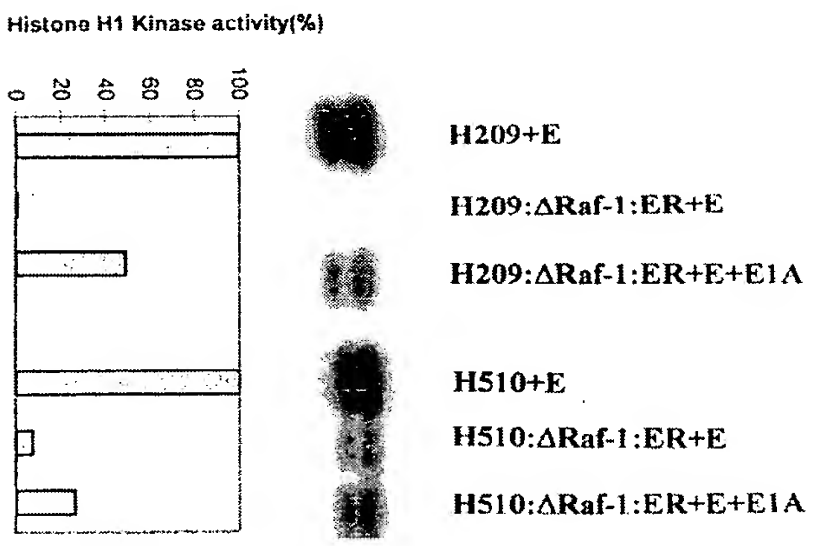


Figure 10. E1A viral oncoprotein restored cdk2 activity in activated ΔRaf–1:ER cells. 50 ng of an E1A 243 amino acid–purified protein (32), were added to 50 μg of activated NCI-H209:ΔRaf-1:ER or NCI-H510:ΔRaf-1:ER cell extracts and incubated on ice for 30 min. cdk2 immunocomplexes were made and kinase activity was determined as described before. Histone H1 phosphorylation was quantitated on a Phosphorimager to calculate the percentage of restoration of cdk2 activity.

kinase pathway as therapeutic targets in the treatment and chemoprevention of SCLC and other neuroendocrine tumors.

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